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14. ABSTRACT

The treatment options for prostate cancer are currently quite limited. Hormonal treatment is the most effective therapy in advanced cancer, however, virtually all the patients who undergo hormonal therapy inevitably develop hormone-resistant tumor. Traditional screening of anti-cancer drugs has been mostly dependent on growth inhibition assay for cancer cells. However, targeting a specific gene with well-defined clinical rationale will provide a better chance of developing a more effective therapeutic agent. We chose a target called Fatty acid synthase (FAS) which we found to be strongly expressed in prostate cancer cells but not in normal cells. Importantly, an inhibition of the FAS expression causes specific tumor cell death. In this project, we plan to test the hypothesis that an active component of *Solidago virgaurea* specifically inhibits the FAS activity and induces apoptosis in prostate tumor cells. Our specific aims are (i) to elucidate the molecular mechanism of growth inhibitory effect of *S. virgaurea* by defining the signal pathway and factors responsible for apoptosis, and (ii) to examine the effect of the active component of *Solidago virgaurea* on tumorigenesis in an animal model.

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INTRODUCTION

The treatment options for prostate cancer are currently quite limited, and no universally accepted therapy is available for patients. Hormonal manipulation is the most effective therapy in advanced cancer in up to 80 percent of patients (1). However, virtually all the patients who undergo hormonal therapy inevitably develop hormone-resistant tumor cells. In the last two decades, numerous chemotherapeutic agents have been studied. However, the overall results have been quite disappointing. The inefficacy of the chemotherapeutic agents on prostate cancer is partly due to the acidic environment and slow growth of the tumor, however, the exact mechanism of the resistance is yet to be understood. The failures of current approaches to develop a new chemotherapeutic agent indicate that we need an essentially new approach to this cancer. Traditional screening of anti-cancer drugs has been mostly dependent on growth inhibition assay for cancer cells. However, targeting a specific gene with well-defined clinical rationale will provide a better chance of developing a more effective therapeutic agent.

FAS is expressed at low or undetectable level in most normal human tissues, with the exception of lactating breast and cycling endometrium. In contrast, elevated expression of FAS and abnormally active endogenous fatty acid synthesis are characteristics of many human cancers, and the up-regulation of FAS was related in most cases to poor prognosis (2,3). Although the biological basis for this phenotype alteration in cancer cells is not clearly understood, it represents an experimental strategy for cancer therapy because inhibition of FAS is selectively cytotoxic for tumor cells and causes apoptosis. How the inhibition of FAS leads to cell death is an intriguing question. Considering that almost none of the conventional chemotherapeutic agents are effective for prostate cancer, we turned our attention to natural and herbal products that have been used for cancer treatment in different geographic areas. After screening over 100 different herbal plants for their inhibitory effect on the FAS expression, we found that *S. virgaurea* has strong suppressor activities on the FAS gene. The cytotoxic activity of *S. virgaurea* appears to be mediated by inhibition of FAS, which eventually leads to apoptosis. The most intriguing question is how *S. virgaurea* suppresses the expression of FAS. We hypothesize that the active component of *S. virgaurea* suppresses tumor growth by inducing apoptosis through inhibition of FAS and that this inhibitory effect on FAS is mediated by blocking the upstream signal of FAS gene expression including PI3, MAPK and Akt. In this project, we plan to accomplish two specific aims: (i) define the mechanism of cytotoxic activity of *Solidago virgaurea,* and (ii) to examine the effect of the active component of *Solidago virgaurea* on tumorigenesis in a transgenic animal model of prostate cancer

BODY

Task 1a: We will first purify the active component of *S. virgaurea* through a series of column chromatography.

We attempted to further purify the cytotoxic activity of *S. virgaurea* using various chromatographic media and found that a combination of heat-treatment followed by column chromatography of G100 and methyl-HIC (BioRad) can effectively purify the activity. The crude extract of *S. virgaurea* was heated at 80°C for 5min followed by centrifugation. The supernatant was concentrated by the Amicon concentrator and applied on a G100 column. The active fraction of G100 was then applied onto the HIC column which was sequentially eluted with 2.4, 1.8, 1.2, 0.8, 0.6 and 0.3M $(NH_4)_2SO_4$. When each fraction was dialyzed and assayed, we found that the cytotoxic activity was eluted with 1.2 M

$(NH_4)_2SO_4$. After repeating the purification steps with G100-sephadex and the HIC chromatography, the final HIC fraction was analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig.2,

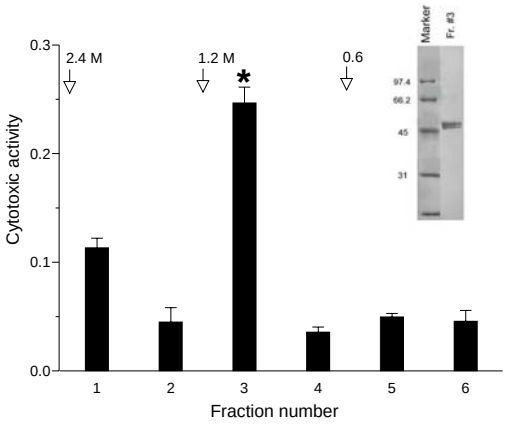

Fig. 1. Purification of cytotoxic activity.
The active fractions of G100 column chromatography were pooled, dialyzed and applied to an HIC column, which was washed and eluted with ammonium sulfate buffer with the indicated salt concentrations. The eluted fractions were assayed for their cytotoxic activities and subjected to SDS-polyacrylamide gel electrophoresis (inset).

the active fraction eluted from the HIC column contained two species of proteins that had molecular weights around 47-49 kD. We have also tried various traditional column chromatographies including DEAE, HA, phosphate, ConA and heparin agarose. However, these column systems did not retain the active component under all tested conditions. We are currently trying to segregate these two proteins by other chromatographical methods including HPLC and Mass spectrometry analysis.

Task 1b: We will examine the status of the FAS signaling pathway upon addition of *S. virgaurea*. We will also examine the expression of various signal molecules using the antibody microarray.

Because the expression of FAS is known to be partly controlled by the Akt pathway, we have examined the effect of *S. virgaurea* on the phosphorylation status of Akt. Human prostate cell line, PC3mm, was cultured in the presence and absence of *S. virgaurea* for 24 hrs. The cells were harvested and the cell lysate was subjected to Western blot using pan- and phospho-specific antibodies (Fig. 2). Our results indicate that *S. virgaurea* indeed strongly inhibited the phosphorylation of Akt as well as the expression of FAS. This inhibition also accompanied by Caspase 3 activation as shown in Fig. 3. These results indicate that inhibition the FAS expression by S. virgaurea is partly due to the blockade of the Akt pathway and that this blocking induces Caspase 3-dependent apoptosis pathway.

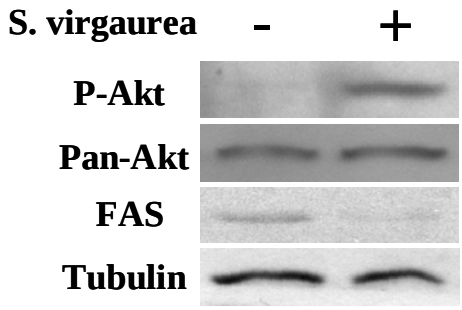

Fig. 2. Inhibition of FAS by S.virgaurea is mediated via Akt pathway. PC3mm cells were treated with or without S. virgaurea for 24 hrs and the cell lysate was subjected to Western blot analysis using antibodies to phosphor-Akt, pan-Akt, FAS and tubulin.

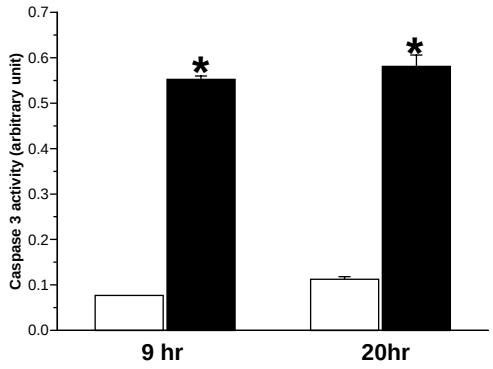

Fig. 3. Caspase assay. PC3 cells (5 x 10^6 cells) were mixed with (closed bar) or without (open bar) the G100 fraction in 1ml of RPMI medium for 9 and 20 hours at 37°C. The cells were then harvested and the cell lysates were tested for the Caspase-3 activity by ApoAlert kit (Clontech). Values are mean +/- SD of triplicate

Task 1c. **We will examine the status of Malonyl-CoA, ceramide and the expression of the pro-apoptotic genes, BNIP3, DAPK2 and TRAIL as well in response to *S. virgaurea*.**

This task is currently ongoing. We have previously shown that inhibition of FAS expression by shRNA accumulated ceramide and induced BNIP3, DAPK2 and TRAIL. We exepct that *S. virgaurea* shows a similar effect on prostate tumor. We have worked out all the technical aspect for these assays and we hope to accomplish this task soon.

Task 2. To examine the effect of the active component of *Solidago virgaurea* on tumorigenesis in a transgenic animal model of prostate cancer

a. We will examine the pharmacokinetic and pharmacodynamic parameters, maximum-tolerated dose and toxicity after administration of the purified protein into mice.
b. We will administer the active component to the TRAMP (transgenic adenocarcinoma mouse prostate) mice and examine the growth of tumor and incidence of metastasis.
c. We will examine the tumor of the animals for the status of the expression of BNIP3, DAPK2 and TRAIL genes as well as apoptotic index.

We have been waiting for the purified compound of Solidago virgaurea before pursuing Task 2. Due to the delay of recruitment of personnel for this project in the first year, we are somewhat behind the schedule. However, we expect to catch up with the pace after these researchers are fully engaged in the project.

While we were waiting for the progress on Task 1, we also pursued a possibility of using another natural product, *Cacalia deliphiniifolia,* for prostate cancer therapy. We have identified anti-FAS activity of *Cacalia deliphiniifolia* when we initially screened various national products for the current project. The results of the screening identified Solidago virgaurea which showed the highest anti-FAS activity as we described in the current project. However, the extracts of *Cacalia deliphiniifolia* also showed strong anti-FAS activity in our in vitro assay. Therefore, to accomplish the overall goal of our project which is to identify natural compounds to block FAS activity, we also decided to study *Cacalia deliphiniifolia* in parallel. As shown in Fig. 4a. the extracts of *Cacalia deliphiniifolia* significantly inhibited the expression of FAS in a prostate cancer cell, PC3mm, in a target specific manner. This inhibition of FAS expression also led to inducing apoptosis measured by TUNEL assay (Fig. 4b).

(A) **(B)**

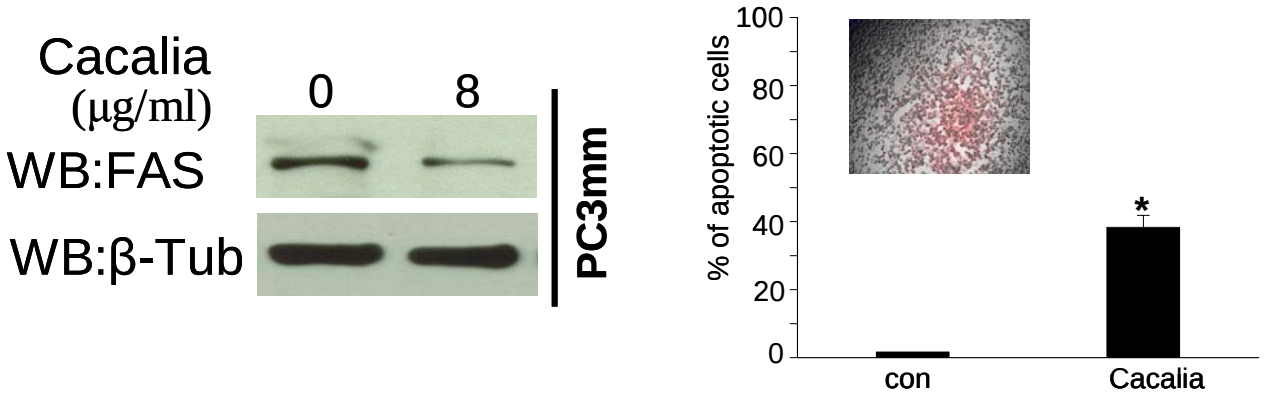

Fig. 4. *Cacalia deliphiniifolia* inhibits FAS expression and induces apoptosis. PC3mm cells were cultured in the presence or absence of *Cacalia* for 24 hrs. Cell extracts were then subjected to Western blot analysis using FAS-specific antibody (A). The cells were cultured in 96-well plate and treated with or without *Cacalia* for 36 hrs. They were then assayed for apoptosis using Cell death TMR kit (B).

KEY RESEARCH ACCOMPLISHMENTS

1. We have found that *Solidago virgaurea* blocks phosphorylation of Akt followed by inhibition of FAS expression.
2. This inhibition follows activation of Caspase 3 and induction of apoptosis.
3. The purification of active component of *Solidago virgaurea* is underway and we will sort out systems that would be helpful for the next stage of purification.
4. We found another natural product, *Cacalia deliphiniifolia* ,which blocks FAS expression and induces apoptosis. We will also pursue this product as a part of this project in the following years.

REPORTABLE OUTCOMES

Peer reviewed publications
(The following works were directly or partly supported by the current grant)

1. Furuta, E., Pai, SK., Zhan, R., Bandyopadhyay, S., Watabe, M., Iiizumi, M., Liu, W., Mo, Y-Y., Hirota, S., Hosobe, S.,Tsukada,T., Miura,K., Kamada, S., Saito, K. and Watabe, K. (2008) Fatty acid synthase gene is up-regulated by hypoxia via activation of Akt and SREBP. *Cancer Res.* 68, 1003

Abstract/presentation

1. Eiji Furuta, Rui Zhan, Sucharita Bandyopadhyay, Shigeru Hirota, Sadahiro Hosobe, Misako Watabe, Sudha K. Pai, Megumi Iiizumi, Sonia Mohinta, Wen Liu, Kounosuke Watabe. Hypoxia induced ROS up-regulates the fatty acid synthase gene via Akt pathway in breast cancer cells. (2008) Annual meeting of American Association for Cancer Research. San Diego, CA

2. Wen Liu, Eiji Furuta, Misako Watabe, Kazutoshi Shindo, Megumi Iiizumi, Sudha Pai, Kounosuke Watabe. (2008) Inhibition of Fatty acid synthase and induction of apoptosis in human breast cancer cells by Cacalia deliphiniifolia Annual meeting of American Association for Cancer Research. San Diego, CA

3. Megumi Iiizumi, Sucharita Bandyopadhyay, Sudha K Pai, Misako Watabe,Shigeru Hirota, Sadahiro Hosobe, Taisei Tsukada, Kunio Miura, Ken Saito, Eiji Furuta, Wen Liu, and Kounosuke Watabe (2008) RhoC promotes metastasis but not growth of prostate tumor. Annual meeting of American Association for Cancer Research. San Diego, CA

Employment

1. Wen Liu (Graduate student) has been supported by the current grant.
2. Dr. Aya Kobayashi (Postdoc) has been partly supported by the current grant.
3. Dr Eiji Furuta (Postdoc) has been partly supported by the current grant.

CONCLUSIONS

During the last funding period, our progress was somewhat behind the schedule due to the initial delay in the recruitment process. However, we expect to catch up with the pace during the next funding period. To accomplish Task1a, we tried many different column systems without much success. We will continue our efforts to find a better way to purify the active component. While pursuing the Task1b, we have found that Akt pathway is involved in the inhibition of FAS by *Solidago virgaurea*. This significant finding opened several avenues to investigate the signal pathways of *S. virgaurea* which lead to apoptosis of tumor cells. We plan to perform the antibody array analysis to obtain further information in the pathway as proposed in the original application during the next cycle. We also found that another natural product, *Cacalia deliphiniifolia*, also showed strong anti-FAS activity in prostate cancer cells. This is particularly interesting because we can expect synergistic effect of *Solidago virgaura* and *Cacalia deliphiniifolia*, on FAS expression which may have significant impact on prevention of prostate cancer by proper diet.

So what?

Prostate cancer is one of the most resistant tumors to chemotherapy among all adenocarcinomas, and there is virtually no effective therapeutic regimen available for this cancer. The failure of the current approach to develop an anti-prostate cancer drug suggests that we need essentially a new approach by defining a specific target molecule in this cancer. Our preliminary data indicate that FAS is considered to be an ideal target for this purpose. *S. virgaurea* has been used as herbal medicine in the past to treat urological diseases and known to be non-toxic. Our discovery of specific inhibition of the FAS activity by the extract of *S. virgaurea* suggests a potential utility of this traditional medicine as a chemopreventive as well as therapeutic remedy for prostatic cancer. During this funding cycle, we obtained an important clue in understanding how *S. virgaurea* inhibits the FAS expression and induces apoptosis. We also found that another natural product *Cacalia deliphiniifolia* also blocks the FAS expression. This exciting finding adds another layer of interest to this project because of the potential utility of both products as non-toxic chemopreventive agents for prostate cancer.

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